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Application of Plant Growth-Promoting Microbes on Urban Building Vegetated Envelopes, from Lab to Field

Long Xie

ACADEMIC DISSERTATION

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Abstract

Urban greening has gained increasing popularity in cities to create a more sustainable, climate-change resistant, and esthetic living environment. In reality, other forms of urban development are given priority over green spaces in densely populated cities. Therefore, the application of vegetated building envelopes (VBEs) might be a solution to resolve the land use conflict.

VBEs refer to all forms of vegetation structures installed to facades or rooftops of buildings. The large-scale use of VBEs in cities can provide multiple ecosystem services, such as managing stormwater, mitigating air pollution, conserving energy, and reducing urban heat island effect. More and more vegetated roofs have been built in the Helsinki region, and they are becoming an important part of urban greening.

However, research on VBEs received limited attention from academia. So far, related studies have been mostly focusing on the identification and quantification of ecosystem services, especially on stormwater management and energy conservation. Moreover, growing conditions for plants on building envelopes are harsh in general, because of the often shallow substrate with relatively low water holding capacity, full exposure to solar radiation and wind (leading to fluctuating temperatures), and nutrient leaching via runoff. It is becoming imperative to investigate how to maintain plants on VBEs at their optimum.

The present Ph.D. project focused on maintaining plant growth under poor growing conditions in VBE systems. The main solution was to inoculate two species of plant growth-promoting microbes in the substrate, i.e., *Rhizophagus irregularis* and *Bacillus amyloliquefaciens*. *R. irregularis* is an arbuscular mycorrhizal fungus (AMF) that can form symbiosis within host plant roots. Typical structures of AMF are hyphae, arbuscules, and vesicles. *B. amyloliquefaciens* is a gram-positive, spore-forming bacterium that forms a bacterial-cell layer called biofilm on the root surface. Both microbes can increase systemic resistance of host plants against a wide range of pathogens and pests, and strengthen host plant tolerance against various abiotic stresses, such as drought, high salinity, heavy

metal contamination, and nutrient deficiency. In return, the host plants support the microbes with root exudates rich in photosynthetic compounds.

This doctoral dissertation consists of three phases. The first phase was to test whether the microbial inoculants could survive in VBEs and successfully colonize a group of plant species (*Fragaria vesca*, *Poa alpina*, *Trifolium repens*, *Thymus serpyllum*, and *Viola tricolor*). The survival and colonization of both microbes were verified, and *R. irregularis* was speculated to increase the bacterial density of *B. amyloliquefaciens*. It also suggested that substrate pH and biochar amendment could influence *R. irregularis* colonization level. The second phase was to confirm whether another group of plant species selected for VBE systems (*Antennaria dioica*, *Campanula rotundifolia*, *F. vesca*, *Geranium sanguineum*, *Lotus corniculatus*, *T. serpyllum*, *T. repens*, and *V. tricolor*) could host the microbes, and to test the effects of single- and co-inoculation of the microbes on plant growth in controlled lab conditions. The results showed that seven out of the eight tested plant species could co-host both microbes, and *R. irregularis* colonization level was improved by the presence of *B. amyloliquefaciens*. Most importantly, the co-inoculation of *R. irregularis* and *B. amyloliquefaciens* led to higher shoot biomass and photosynthetic efficiency than separate single-inoculation. The last phase was to verify whether the co-inoculation effect produced from the lab would repeat on vegetated roofs, and which plant species, planting methods, and their interactions would produce the best plant and microbial growth on vegetated roofs. The results confirmed plant growth-promotion via co-inoculation on vegetated roofs. Moreover, *R. irregularis* colonization level was affected by plant species, planting methods and their interactions, while *B. amyloliquefaciens* bacterial density was affected only by plant species.

This doctoral dissertation provides valuable references to build and maintain more stress-tolerant and vigorously growing plants on VBEs, in the hope of making VBE application more affordable and widely used.

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This work was conducted at the Department of Agricultural Sciences, University of Helsinki during 2015-2019. I received enormous support and guidance from my supervisors Professor Jari Valkonen and Docent Susanna Lehvävirta. Therefore, my deepest appreciation goes to my beloved supervisors. My special thanks go to my follow-up group members, Sari Timonen and Motomu Akita, who have offered their support and advice throughout my PhD research.

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I **Xie L**, Valkonen JP, Kuoppamäki K, Hagner M, Jauni M, Lehvävirta S. Effect of weather conditions, soil pH, biochar amendment, and plant species on two plant growth-promoting inoculants on vegetated roofs and facades. Manuscript.

II **Xie L**, Lehvävirta S, Timonen S, Kasurinen J, Niemikapee J, Valkonen JP. Species-specific synergistic effects of two plant growth-promoting microbes on green roof plant biomass and photosynthetic efficiency. PloS one. 2018; 13(12):e0209432.

III **Xie L**, Lehvävirta S, Valkonen JP. Case study: planting methods and beneficial substrate microbes affected the initial growth of vegetated roof plant in Finland. Submitted to Urban Forestry & Urban Greening.

Authors' contribution

Effect of weather conditions, soil pH, biochar amendment and plant species on two plant growth-promoting inoculants on vegetated roofs and facades

Long Xie (LX), Jari Valkonen (JV), Sari Timonen (ST), Kirsi Kuoppamäki (KK), Marleena Hagner (MH), Susanna Lehvävirta (SL).

LX, SL and JV designed the first and second independent experiments, and KK and MH designed the third independent experiment. LX collected all the samples. LX conducted all the lab works, including microbial inoculation in field and lab, plant cultivation in growth chamber, DNA extraction from soil and inoculant product, PCR and DNA sequencing, qPCR, root sample staining, and AMF quantification by microscopy. LX conducted data analysis, visualization and composed the original manuscript. LX, ST, SL and JV revised the manuscript.

Species-specific synergistic effects of two plant growth-promoting microbes on green roof plant biomass and photosynthetic efficiency

Long Xie (LX), Susanna Lehvävirta (SL), Sari Timonen (ST), Jutta Kasurinen (JK), Juhamatti Niemikapee (JN), Jari Valkonen (JV).

LX, SL and JV designed the greenhouse experiment. LX cultivated the plants in NaPPI, conducted microbial inoculation, computer programming for measurements, and carried out daily maintenance. LX collected root, soil, and plant shoot samples. LX and JK performed root staining, microscopy analysis, and quantification of AMF abundance. LX performed plant shoot dry biomass quantification, DNA extraction from soil and inoculant product, PCR, DNA sequencing, and qPCR quantification. LX designed and modified bacterial quantification based on qPCR. LX conducted data analysis, visualization and composed the original manuscript. LX, JN, ST, SL and JV revised the manuscript.

Case study: planting methods and beneficial substrate microbes affected the initial growth of vegetated roof plant in Finland

Long Xie (LX), Susanna Lehvävirta (SL), Jari Valkonen (JV).

LX, SL and JV designed the field experiment on a rooftop of a residential building. LX inoculated two vegetated roofs with selected microbial inoculants. LX collected soil, root, and plant shoot samples from the vegetated roofs. LX quantified plant growth with shoot dry weight. LX extracted DNA from soil samples and inoculant product for DNA sequencing, conducted qPCR to determine bacterial population density. LX quantified AMF abundance using root staining and microscopy techniques. LX conducted data analysis, visualization and composed the original manuscript. LX, SL and JV revised the manuscript.

Abbreviations

AMF	Arbuscular mycorrhizal fungus (fungi)
GFP	Green fluorescent protein
H_2O_2	Hydrogen peroxide
HSDH	Heat stress degree hour
HCl	Hydrochloric acid
K	Potassium
KOH	Potassium hydroxide
NaPPI	National Plant Phenotyping Infrastructure
N	Nitrogen
NH_3	Ammonia
MHB	Mycorrhiza helper bacterium
P	Phosphorus
PCR	Polymerase chain reaction
PGPM	Plant growth-promoting microbe
PGPR	Plant growth-promoting rhizobacteria
qPCR	Quantitative polymerase chain reaction
RGB	Red, green, blue
ROS	Reactive oxygen species
VBE	Vegetated building envelope
VOC	volatile organic compound
WHC	Water holding capacity

1. Introduction

Vegetated building envelopes (VBEs), typically vegetated roofs and facades, are urban landscapes incorporating multiple expertise in architecture, agriculture, material, ecology, design, and policies. Compared to asphalt- or tar-pasted roofs, vegetated roofs have been well-documented to provide multiple ecosystem services to ambient urban environments, such as mitigating heat island effect, managing stormwater, conserving energy, alleviating air pollution, and providing experiential pleasure (Shafique et al., 2018; Mesimäki et al., 2019).

Thanks to these ecosystem services, VBE applications have been gaining worldwide popularity in urban planning, especially in densely built areas where more green spaces are needed to deliver ecosystem services (Besir and Cuce, 2018). In recent years, policies such as incentives, guidelines, and coercive regulation have also been made to encourage real estate developers to install vegetated roofs and walls on newly built or retrofitted complexes (Carter and Fowler, 2008). In Finland, VBEs are still rare and mostly built in the Helsinki region. No registers are available about their initial vegetation or roof characteristics (Gabrych et al., 2016). However, more and more new buildings and planning constructions incorporate vegetated roofs and facades across Finland. The trend will continue when more VBEs are built, and they attract the attention of urban dwellers and planners.

Adverse growing conditions on VBEs for vegetation have been widely confirmed and acknowledged (Snodgrass and Snodgrass, 2006). Aloisio et al. (2017) recorded a 60% plant mortality in less than 2 years on vegetated roofs in New York City. Low plant survival rates occurred in other North American cities and was dependent on climate conditions (Tran et al., 2019). Difficulties in cultivating and maintaining VBE plants have been identified in related studies, but few solutions are proposed and investigated to improve the situation. All the facts and difficulties signify the importance of conducting VBE research on how to establish and maintain plants at their optimal growth and development.

Rhizophagus irregularis and *Bacillus amyloliquefaciens* are two reliable plant growth-promoting microbes (PGPM) whose beneficial effects and underlying mechanisms have been extensively investigated (details in 1.3.3 and 1.4.2). In the present project, they were single- and/or co-inoculated with a group of selected plants in greenhouses and VBEs to reveal their survival and impacts on host plants in a relatively new and important urban greening infrastructure.

1.1 Vegetated building envelopes (VBEs)

VBEs refer to building facades or roofs that are covered with vegetation and growth substrates, including vegetated roofs, balcony gardens, and living walls (Djedjig et al., 2015). In earlier modern urban planning, VBEs were built mainly because of their esthetic and recreational values. Only until recent decades, comprehensive research revealed that VBEs could deliver much more ecosystem services than merely looking good. These supporting results from scientific research further promote the utilization of VBEs in urban areas.

1.1.1 Components of VBEs

Here, a vegetated roof is illustrated as an example of VBE components (Besir and Cuce, 2018) (Fig 1). The layers include root barrier and protection layer, drainage layer, filter layer, and growing substrate. Sometimes the layers can be arranged in a different order and some of the layers can be even removed depending on specific designs and functions. The vegetation layer is usually made up of plant species tolerant to drought, direct radiation, heat, and nutrient-poor substrates.

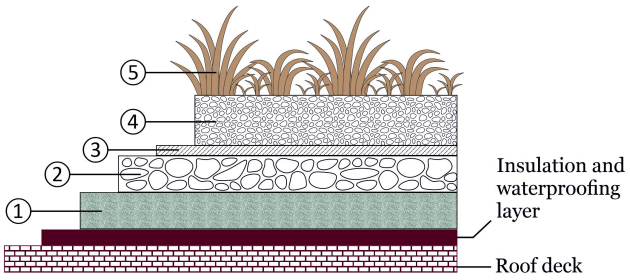

Fig 1. Cross-section of vegetated roof layers. ① Root barrier and protection layer; ② Drainage layer; ③ Filter layer; ④ Growing substrate; ⑤ Rooftop vegetation.

There are several methods to introduce plants onto VBEs, i.e., vegetation mats, plug plants, plants grown from seeds, plant cuttings (sedum), modular systems, spontaneous plants via arrival from the surroundings or the substrate, especially when other natural soil is used as substrate (Emilsson, 2008; Gregoire and Clausen, 2011; Gabrych et al., 2016). The selection of suitable methods should be based on roof load capacity, customer expectations, budget, plant species, and environmental conditions. For example, in climates with high wind speed, pre-grown mats or module systems may be suitable to withstand erosion, and tree seedlings on a vegetated roof should be removed to avoid penetrating the waterproof layer by the roots and exceeding load-carrying capacity.

1.1.2 Ecosystem services and disservices from VBEs

VBEs can provide ecosystem services, but also induce ecosystem disservices if not properly installed and maintained. Vegetated roofs deliver six major benefits: stormwater management, roof protection, energy conservation, heat island effect mitigation, wildlife habitat provision, and elevation of esthetic and market value (McIntyre and Snodgrass, 2010). However, runoff from vegetated roofs may contain a high level of nutrients which can lead to eutrophication of downstream waterbodies (Kuoppamäki and Lehvävirta, 2016). It is likely to happen when vegetated roof developers tend to apply excessive fertilizer to ensure the growth of the plants. As a result, newly built vegetated roofs can cause eutrophication more than old ones (Buffam and Mitchell, 2015). Furthermore, when a vegetated roof is poorly maintained, i.e., the substrate surface is exposed to open air because of low vegetation coverage, the vegetated roof will turn into a source rather than a solution of air pollution. It would spread fine particles during dry and windy days, or leak the fine particles into the runoff, returning into the atmosphere eventually (Tan and Sia, 2005). Other disservices might bring damage to the buildings if the VBEs are not properly designed, installed and maintained, such as leakage and overload with heavy snow (Gartner, 2009; Shafique et al., 2018). Therefore, it is essential to offer evidence-based guidelines for the establishment and maintenance of VBEs to maximize the ecosystem services, while minimizing the potential disservices.

1.1.3 Status and challenges of VBE research

Although VBE is still a relatively new research focus, the last two decades have witnessed a significant increase in VBE studies. Multiple ecosystem services delivered by VBEs is a driving force (Shafique et al., 2018). Such driving force is also shifting the focus of VBE research from architecture and engineering to plant sciences, urban ecology and ecosystem services, especially to ecosystem services (Blank et al., 2013). To overcome the harsh growing conditions on VBEs, two common solutions are adopted: lightweight substrates and drought-tolerant *Sedum* species (Snodgrass and Snodgrass, 2006). Lightweight substrates ensure thicker substrate layers within limited roof load-carrying capacity. Thicker substrate layers can generally provide plants with more nutrients and water than sallow ones, supporting a more diverse plant community (Abd-Elmabod et al., 2017; Shafique et al., 2018), and stabilizing substrate temperature to resist heat stress (Savi et al., 2016). However, the application of lightweight substrates and *Sedum* species might cause some other issues. Many lightweight substrates are heavy in carbon footprint during material processing, such as expanded shale and clay aggregate. Large scale use of such lightweight substrates offsets the benefit of carbon sequestration by VBEs (Chenani et al., 2015; Matlock and Rowe, 2016). Sedum roofs are dominated by sedums and mosses, which may likely turn into moss-dominate roofs later on (Gabrych et al., 2016). As a result, sedum roofs lose part of biodiversity and consequently diminish their ecosystem services to a certain degree (Cook-Patton and Bauerle, 2012). According to Dearborn and Kark (2010), there are many benefits to conserve urban biodiversity, from nature to human needs. Losing urban biodiversity results in climate change, reduced resilience, reduced ecosystem services, and degraded urban areas (Zari, 2018). Additionally, diverse plant species on VBEs brings higher canopy density than monoculture (Tran et al., 2019). Therefore, new solutions should be identified to better support the growth of various plant species on VBEs.

Substrate microbial community studies have been extensively carried out in different ecological and agricultural systems worldwide. These studies aim to understand the dynamics and functions of substrate microbial community, and eventually employ the knowledge to enhance plant growth, biocontrol, substrate remediation, and nutrient cycling (Trabelsi and Mhamdi, 2013). Similarly, the

microbial community on vegetated roof substrate has been surveyed and investigated. For instance, Rumble (2013) reported that microbial community on vegetated roofs is low in abundance, and also adapts to roof growing conditions. Another study conducted in New York City by McGuire et al., (2013) exhibited that not only was the microbial community in roof substrate compositionally distinct from ground level counterparts (urban parks and gardens) but also the roof microbial assembly among individual roofs was locally variable. Furthermore, researchers have applied various PGPMs onto vegetated roofs to test their viability and impact on roof plants. Yet, the outcomes were not consistent, and the impacts were not always positive. Molineux et al. (2014) reported a microbial biomass reduction when AMF and bacterial inoculants were applied together on a vegetated roof site. Young et al., (2015) recorded a successful inoculation of AMF products on vegetated roof modules that increase leaf P content, but not increase leaf N content or plant biomass. According to a vegetated roof field experiment that applied microbial inoculant to test its impact on the substrate food web, Rumble and Gange (2017) hypothesized that the competition between commercial inoculants and incumbent microbes might reduce the success of inoculation, consequently reducing expected beneficial effects.

Despite the fast-growing number of microbial manipulation studies on vegetated roofs, there are limitations in current research. Firstly, most inoculants used were unspecified inoculant mixture, such as compost tea (Molineux et al., 2014), and microbial compatibility was unknown. As a result, expected outcomes might be reduced by microbial competition and suppression. Secondly, the inoculation effect on plant growth/performances was seldom tested on non-succulents on VBEs. Therefore, inoculating specified and compatible PGPMs with forb plant species on VBEs would be more likely to improve plant growth on VBEs and create more biodiverse VBE systems.

In reality, conducting microbial experiments on VBEs is constrained by many conditions. For instance, it is usually not allowed to collect root samples from vegetated roofs for belowground biomass evaluation, which greatly disturbs the plant community and destroys the integrity of the VBEs. For the same reason, a large number of shoot sampling for aboveground biomass evaluation is also restricted. Thus, the experimental replicate number is limited. Additionally, VBE

systems usually have small areas, while experimental plots should be kept distant from each other to avoid contamination. Therefore, the number of microbial treated plots is limited. Moreover, the flow direction of stormwater, which is determined by roof structures, should be considered in placing experimental plots to avoid contamination via runoff. So, the experimental plots might not be completely randomized. However, despite all the limitations, microbial inoculation on VBEs possesses great benefits that need to be revealed by scientific experiments.

1.2 Plants selection for vegetated building envelopes

When selecting plants for VBEs, there are many criteria to take into consideration. Via these criteria, the establishment of VBEs would be more successful.

Firstly, plants that are tolerant of drought, heat, wind, radiation and infertile substrates will be ideal choices for VBEs. Selecting suitable plants that are stress-tolerant to such an adverse growing condition is a major challenge (Arabi et al., 2015). Secondly, plant selection should be in line with engineering and architectural requirements. For instance, tree seedlings should be constantly removed from vegetated roofs that are not designed to accommodate trees. Unwanted tree seedlings would produce aggressive roots to search for water, which will damage the building envelope (Miller et al., 2014). Other plants with strong root systems should also be excluded to avoid waterproofing layer penetration, which might result in leakage. Thirdly, it is a good practice to introduce native plant species to VBEs. Native plant species might be easier to adapt to the growing conditions on VBEs, even though it does not guarantee survival (Tran et al., 2019). Moreover, using native plant species eliminate the possibility of introducing invasive plants (MacIvor and Lundholm, 2011). Lastly, VBEs are also an important source of urban diversity (Dearborn and Kark, 2010; Zari, 2018; Tran et al., 2019). A variety of plant species on VBEs are preferred to have higher biodiversity, horticultural value, and esthetic values.

The plant species used in this project are all native and non-invasive species in Finland according to NatureGate (http://www.luontoportti.com/suomi/en/) and

Global Invasive Species Database (http://www.iucngisd.org/gisd/). The used plant species included ornamental, berry and grass plants that deliver high esthetic and horticultural values (i.e., *Antennaria dioica*, *Campanula rotundifolia*, *Fragaria vesca*, *Geranium sanguineum*, *Lotus corniculatus. Thymus serpyllum*, *Trifolium repens*, and *Viola tricolor*). They are herbaceous plant species that do not develop strong root systems to penetrate protection and waterproofing layers, and they are highly stress-tolerant to abiotic stresses, such as drought and heat, which makes them suitable for growing conditions on VBEs (Lewis, 1969; Taschler and Neuner, 2004; Striker et al., 2005; Stevens and Wilson, 2012; Moradi et al., 2014; Kipkeev et al., 2015). Most importantly, their compatibility with mycorrhiza has been confirmed. For instance, Scheublin et al. (2004) reported that *Glomus intraradices* colonization was more frequent in legumes plants, i.e., *L. corniculatus* and *T. repens*, than nonlegumes. *A. dioica* was found to be colonized by *G. intraradices*, which was affected by nitrogen content in the substrate (Santos-González et al., 2007). *F. vesca* and *V. tricolor* were also reported as host plants for *R. irregularis* (Sinclair et al., 2014; Zubek et al., 2015).

1.3 *Rhizophagus irregularis*

R. irregularis belongs to arbuscular mycorrhizal fungi (AMF) and distributes in almost all substrate types (Strack et al., 2003). *R. irregularis* was previously named as *Glomus intraradices* but then renamed *Glomus irregulare* as it was identified to be a separate species (Stockinger et al., 2009). Recently, *G. irregulare* was renamed *R. irregularis* again after a more accurate phylogenetic characterization (Krüger et al., 2012). After successful colonization in the roots, *R. irregularis* can facilitate the host plants to take up essential nutrients, increase tolerance to abiotic stresses, and induce systemic resistance against pathogens, in exchange for photosynthetically fixed carbon.

1.3.1 Structures of *R. irregularis* in root systems

The most prominent structure of AMF is the highly branched, tree-like arbuscule in plant root (Fig. 2). Arbuscules are considered as the most essential AMF

structures where the exchange of carbohydrates, minerals, and other nutrients takes place between the fungus and host plants (Strack et al., 2003). Another distinctive structure of AMF is the oval-shaped vesicle that stores lipids. Besides, vesicles have been found to act as propagules that significantly improve the infectivity of plant roofs (Müller et al., 2017). AMF spores resemble vesicles in size and shape, but spores are more spherical than oval, and AMF spores are produced extraradically in the rhizosphere (Pfeffer et al., 1999; Müller et al., 2017). Internal and intraradical hyphae spread inter- and intracellularly in root tissues, functioning as nutrient transportation ducts that connect arbuscules, vesicles, and spores. Extraradical hyphae extend from root tissues into the rhizosphere, which not only enlarges the absorption surface areas of plant roots but also act as environmental sensors (Bago et al., 1998; 2004).

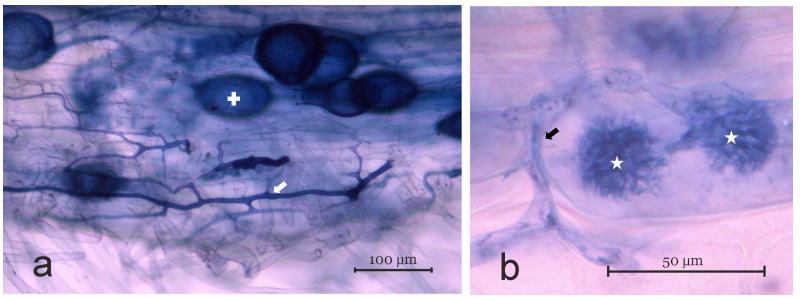

Fig 2. Microscopic images of *R. irregularis* in the roots of *T. repens* (a) and *V. tricolor* (b). Stars, crosses, and arrows indicate arbuscules, vesicles, and hyphae, respectively.

AMF colonization can affect the morphology and function of both host plants and the fungi. For example, even though arbuscule is an intracellular structure, it is surrounded by a peri-arbuscular membrane, separating arbuscule from the cell protoplast. Other morphological changes in host plants during mycorrhization process include major vacuole fragmentation, increase in organelle number, increase in nucleus volume, and nucleus migration from the periphery to the center of host cells (Strack et al., 2003).

1.3.2 Development of *R. irregularis* in host plants

The colonization process starts with the germination of *R. irregularis* spore, which is suggested to be triggered by secondary plant compounds released in the

substrate, leading to plant-microbe interaction and recognition. The most notable signaling compounds are flavonoids and strigolactones, acting as chemoattractants (Steinkellner et al., 2007). Moreover, plant cytoskeletal components, i.e., microtubules and microfilaments, have been verified to play central roles in the exchange of signaling compounds between host plants and fungi and changing host cell morphology and architecture upon colonization (Blancaflor et al., 2001; Timonen and Peterson, 2002).

When the spore hypha reaches the host plant root, the appressorium is formed on the tip of the hypha and attached to the root surface (Fig. 3). Later, the appressorium initiates penetration into the epidermal cells by increasing turgor pressure with the help of non-aggressive cell wall lytic enzymes (Chang et al., 2014). Afterward, the colonization process continues with the spreading of inter- and intracellular hyphal net in the root tissues, which may later differentiate into arbuscules or vesicles (Lambais, 2006). Arbuscules are short-lived structures that only last 4 to 10 days before senescence and collapse (Sanders et al., 1977). Extraradical hyphae protrude from root tissues and extend into the rhizosphere. Eventually, spores are formed on the extraradical hyphae and enter another cycle of AMF colonization (Bago et al., 1998). In some cases, vesicles can also act as propagules (Müller et al., 2017).

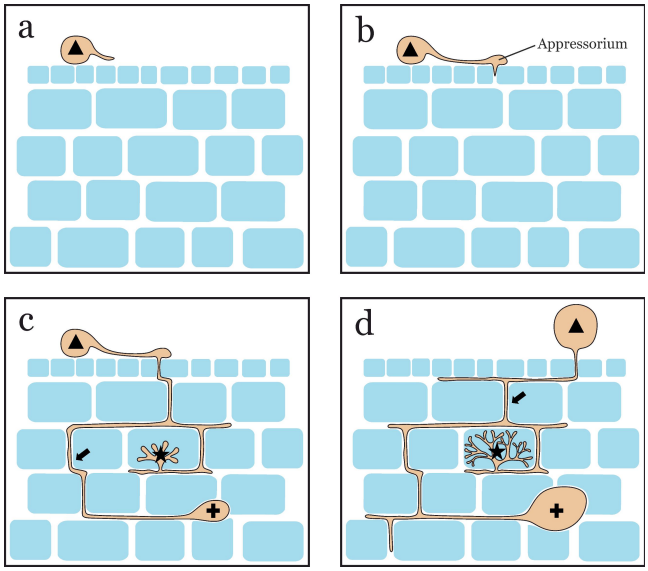

Fig 3. AMF colonization process recreated based on Strack (2003). Spore firstly germinates on the surface of host plant root (a); then appressorium is formed on the tip of the hypha and anchors at the entry point for penetration (b); next, the hyphae spread in the root tissues, and arbuscules and vesicles appear (c); lastly, spores are produced ready for the next cycle of colonization (d). Stars, crosses, arrows, and triangles indicate arbuscules, vesicles, hyphae, and spores, respectively.

1.3.3 Benefits of *R. irregularis* application

Experiments have shown that *R. irregularis* increase nutrient uptake of host plants (George et al., 1995; Koide and Kabir, 2000). Phosphorous (P) absorption by *R. irregularis* may partially contribute to a high phytase production that can hydrolyze indigestible organic P. For instance, it has been found that *Daucus carota* plants inoculated with *R. irregularis* can produce more phytase than non-inoculated ones. However, it is not clear whether *R. irregularis* releases phytase production from itself or stimulates the plants to produce more phytase (Koide and Kabir, 2000). Additionally, *R. irregularis* may form a unique fungal-bacterial symbiosis with phosphate solubilizing bacteria via extraradical hyphae exudates. By supporting the growth and activities of such bacteria, both AMF and host plants benefit from their P solubilizing activities (Taktek et al., 2015).

R. irregularis inoculation can also counteract the effect of abiotic stresses caused by high salinity and drought in the substrate. There are various mechanisms proposed to explain the outcome. In earlier studies, alleviation of drought and salt stresses by *R. irregularis* appeared to be achieved by regulating the physiological process of host plants (Ruiz-Lozano et al., 1995; 1996). Later, research revealed that *R. irregularis* could maintain hydraulic conductivity under drought and salinity stresses in both plant leaves and roots by regulating related gene expression (Aroca et al., 2007).

The last but not the least commonly acknowledged benefit of *R. irregularis* inoculation is resistance to biotic stress. *R. irregularis* has been intensively verified on a large variety of host plant species to induce systemic resistance against a wide range of microbial pathogens and pests (Xavier and Boyetchko, 2004). Whipps (2004) concluded that the mechanisms of pathogenic resistance include competition for resources, enhanced plant growth, and inhibiting biochemical production.

1.4 *Bacillus amyloliquefaciens*

Some *Bacillus* species have been repeatedly reported to produce phytohormones to regulate plant growth, and antibiotics to control microbial pathogens. These beneficial members are also known as plant growth-promoting rhizobacteria (PGPR) (Kloepper et al., 2004; Sumi et al., 2014). Among the *Bacillus* species, *B. amyloliquefaciens* is one of the most intensively studied ones. Therefore, *B. amyloliquefaciens* has been commercially manufactured and utilized for agricultural applications (Fravel, 2005).

B. amyloliquefaciens is closely related to a model organism, i.e., *Bacillus subtilis*, and it has been difficult to distinguish them only from phenotypic features and classical tests. Because of that, *B. amyloliquefaciens* was earlier categorized as a subspecies of *B. subtilis* (Tsuru, 1962). The name of *B. amyloliquefaciens* comes from the fact that it produces α-amylase and protease (Fukomoto, 1943). Three decades ago, Priest (1987) pointed out that the two species differ in metabolism pathways, enzyme production, and most importantly, DNA sequence, using several new techniques back then. And gradually, *B. amyloliquefaciens* was accepted as a separate *Bacillus* species and has been studied as a model bacterium of *Bacillus* species in plant-microbe interactions (Chowdhury et al., 2015).

1.4.1 Structure and life cycle of *B. amyloliquefaciens*

B. amyloliquefaciens is a gram-positive, and rod-shaped bacterium. The bacterial cell measures 0.7 to 0.9 μm wide by 1.8 to 3.0 μm long. The oval spore measures 0.6 to 0.8 μm wide by 1.0 to 1.4 μm long. The bacterial cells feature flagella to render their mobility (Priest, 1987).

Attracted by plant root exudates, *Bacillus* species densely populate in the rhizosphere, a process known as tropotaxis (Kierul et al., 2015). The successful establishment of PGPR-plants interaction involves mutual recognition and mechanisms that evade the innate immune system of host plants (Pieterse et al., 2012). After the recognition process, the bacterial cells eventually anchor themselves to the root surfaces (Reva et al., 2004).

The life cycle of *Bacillus* species has two distinct parts: sporulation cycle and vegetative cycle (Errington, 2003) (Fig. 4). The vegetative cycle happens when growing conditions are favorable. The mother cell divides equally into two viable daughter cells and cell density increases exponentially. When growing conditions become adverse, the sporulation cycle occurs, producing endospores. The endospores are resistant to extreme environments. When encountering suitable conditions, the endospores will germinate, colonize host plants, and propagate through new a vegetative cycle (Nicholson et al., 2000).

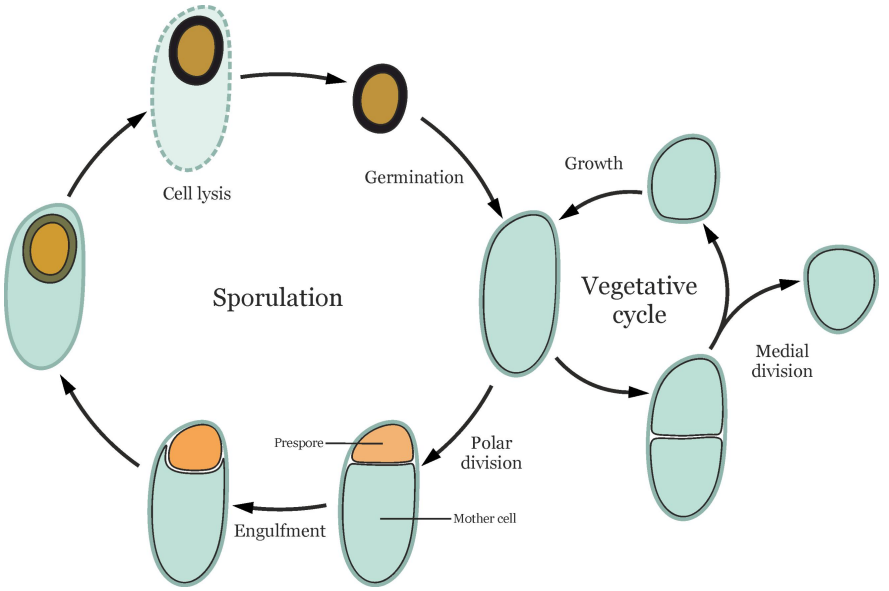

Fig 4. The life cycle of *Bacillus* species recreated based on Errington (2003). The typical life cycle of spore-forming *Bacillus* species involves two cycles: sporulation cycle (left) and vegetative cycle (right).

1.4.2 Benefits of *B. amyloliquefaciens* application

Formation of a layer of bacterial cells on the root surface, known as biofilm, is a prerequisite for a successful symbiosis between *B. amyloliquefaciens* and compatible host plants. This biofilm not only synthesizes antibiotics and phytohormones but also prevents competition from other microbes in the substrate (López et al., 2009). Similar to *R. irregularis*, *B. amyloliquefaciens* can

induce systemic resistance against pathogens and increase host plant resilience to abiotic stresses through various mechanisms.

B. amyloliquefaciens can also produce extracellular phytases in phosphate limited substrates, which can hydrolyze phytate to inorganic phosphate, making it a phosphate solubilizing microbe. Idriss et al. (2002) experimented with wild type *B. amyloliquefaciens* FZB45 that can produce phytase and mutant strain FZB45/M2 that could not. They found that plants inoculated with FZB45 increased significantly higher in shoot weight, root weight, and root length than plants inoculated with FZB45/M2, by 42.3%, 40.8%, and 24.5%, respectively.

Drought stress alleviation has been observed in a wide range of host plant species inoculated with *B. amyloliquefaciens*. For example, maize seedlings were inoculated with a group of *Bacillus* species (including *B. amyloliquefaciens*) separately, and showed increased biomass, relative water content and leaf water potential under drought stress, compared with non-inoculated plants (Vardharajula et al., 2011). The underlying mechanism behind it has been attributed to regulating the expression of drought stress-related genes by *B. amyloliquefaciens* inoculation (Tiwari et al., 2017).

B. amyloliquefaciens can provide host plant protection against biotic stresses, e.g. fungi, bacteria, viruses, and pests. There are two major mechanisms behind the biocontrol feature. For one thing, *B. amyloliquefaciens* can produce metabolites as antibiotics to directly lyse cell walls and membranes of substrate-borne pathogens (Chowdhury et al., 2015). For another, *B. amyloliquefaciens* can elicit and enhance host plant immunity with its bacterial metabolites. The protection is systemic, meaning *B. amyloliquefaciens* colonizing roots defends against pathogenic attacks on the shoots (Kloepper et al., 2004).

1.5 Innovation of the present project

R. irregularis and *B. amyloliquefaciens* have been manufactured as substrate additives and applied to promote agricultural production. However, this nature-

based solution was seldom tested in other ecological systems, such as VBEs. In this Ph.D. project, a series of indoor and outdoor experiments were conducted to test the validity of using PGPMs in VBEs by evaluating the inoculation impact on plant performances and microbial population. The project was aimed to establish and maintain flourishing and stress-tolerant VBE plants. This project also provided a standard protocol to select beneficial plant-microbe combinations from lab to field. Hopefully, this project would draw more attention from academia to VBE research, and the practice of collective knowledge would lead to more affordable and fully functional VBEs, providing better ecosystem services to urban residents.

2. Hypotheses

PGPM inoculation in VBE substrates to manipulate substrate microbial community is a relatively new and cost-effective method to improve plant growth and survival on VBEs.

The three publications (**I**, **II**, **III**) collectively tested the following hypotheses:

I, **II**, **III**	*R. irregularis* and *B. amyloliquefaciens* are compatible with a wide range of host plant species.
I, **II**, **III**	The microbial population is affected by host plant species, planting methods, substrate temperature/moisture, substrate pH, and biochar amendment.
I, **II**	*R. irregularis* and *B. amyloliquefaciens* help the growth of each other.
II, **III**	Plant growth is promoted by single- and co-inoculation of *R. irregularis* and *B. amyloliquefaciens* in both lab and field conditions.

3. Materials and methods

3.1 Biological materials

Table 1. Plant, microbial inoculant, and other materials used in this project.

Plants/microbial inoculants	Publication(s)	Provider(s)
Antennaria dioica (seeds)	**II**	Suomen Niittysiemen Oy
Campanula rotundifolia (seeds)	**II**	Suomen Niittysiemen Oy
Fragaria vesca (seeds)	**II**, **III**	Suomen Niittysiemen Oy
Fragaria vesca (plug plants)	**II**, **III**	Terolan Taimitarha Oy
Geranium sanguineum (seeds)	**II**	Suomen Niittysiemen Oy
Lotus corniculatus (seeds)	**II**	Suomen Niittysiemen Oy
Poa alpina (seeds)	**I**	Suomen Niittysiemen Oy
Thymus serpyllum (seeds)	**II**, **III**	Suomen Niittysiemen Oy
Thymus serpyllum (plug plants)	**II**, **III**	Terolan Taimitarha Oy
Trifolium repens (seeds)	**I**, **II**, **III**	Suomen Niittysiemen Oy
Trifolium repens (plug plants)	**II**, **III**	Terolan Taimitarha Oy
Viola tricolor (seeds)	**I**, **II**, **III**	Suomen Niittysiemen Oy
Viola tricolor (plug plants)	**II**, **III**	Terolan Taimitarha Oy
Sedum vegetation mat	**I**	Veg Tech AB
Meadow vegetation mat	**I, III**	Terolan Taimitarha Oy
MYC4000 (*R. irregularis*)	**I, II, III**	Lallemand Plant Care
Rhizocell (*B. amyloliquefaciens*)	**I, II, III**	Lallemand Plant Care
Growth substrates	**I, III**	Hyvinkään Tieluiska Oy
Growth substrate	**II**	Kekkilä Oy
Natural compost	**I**	Biolan Ltd
PowerSoil DNA Extraction Kit	**I, II, III**	Mo Bio/QIAGEN
DNeasy Plant Mini Kit	**I, II, III**	QIAGEN
LightCycler® 480 SYBR Green I Master	**I, II, III**	Roche

3.2 Detection of *R. irregularis* in root samples

In the three publications, detection of *R. irregularis* colonization level was conducted according to the same protocol. The protocol was based on a modified root staining method and the gridline intersect method (Phillips and Hayman, 1970; McGonigle et al., 1990).

In the first step, the root samples were stained and made into microscopic slides. In general, the root samples were first soaked in the KOH solution and then transferred into the hydrogen peroxide solution containing ammonia (H_2O_2+NH_3). Then the root samples were neutralized in HCl solution and held in the heated trypan blue solution for staining. According to plant species, the procedure might be slightly different. Later, the root samples were mounted on microscope slides in polyvinyl-lacto-glycerol solution (10 ml/l water, 10 ml/l lactic acid, 1 ml/l glycerol, and 1.66 mg/l polyvinyl alcohol). In the second step, the stained root sample slides were observed using a microscope. There was a fine crosshair mark on the ocular. When moving the slide in the vertical direction, AMF structures were recorded at the point where the vertical hair intersected a root. There are eight categories: hypha, vesicle, arbuscule, hypha+arbuscule, hypha+vesicle, arbuscule+vesicle, hypha+vesicle+arbuscule, and none AMF structure. In total, 100 intersections were made for each slide. The abundance of each AMF structure was calculated by dividing the sum of positive observation by 100. Detailed process and calculation can be found in original publications (**I, II, III**).

3.3 Detection of *B. amyloliquefaciens* in growth substrate samples

In the three publications, the detection of *B. amyloliquefaciens* bacterial density was conducted according to the same protocol. The protocol was based on PCR and qPCR techniques (Yamamoto and Harayama, 1995; Pfaffl, 2001).

Firstly, DNA samples from both the substrates and the Rhizocell product were extracted using specific DNA extraction kits. PCR amplification was conducted

using primer pair BaG3F (5´-GTCGACCACTCTTGACGTTACGGTT-3´) and BaG4R (5´- CGATCACTTCAAGATCGGCCACAG-3´) to amplify a 94 bp size fragment of the *gyrB* gene. Encoding the subunit B protein of DNA gyrase, the *gyrB* gene is ubiquitous in bacteria for its essential role in the DNA replication process. Therefore, the *gyrB* gene is commonly used as a phylogenetic marker to monitor the bacterial density of *Bacillus* species (Dzieciol et al., 2013). Then the PCR products were sequenced to verify *Bacillus* species. Afterward, the *gyrB* gene amount in soil DNA samples was quantified by qPCR, using Rhizocell product DNA samples as standard. In the end, the bacterial density was shown as the mount of the *gyrB* gene in one gram of substrate (ng/g). Detailed process and calculation can be found in original publications (**I, II, III**).

3.4 Experimental layouts

The first publication (**I**) includes three independent experiments. The first experiment was conducted on a vegetated roof on a retail shop building. *R. irregularis* and *B. amyloliquefaciens* were separately applied in random plots on the roof which was constructed with vegetation mats. The vegetation mats were mainly covered with *P. alpina* and *Sedum* species, and *P. alpina* plants were sampled to detect the microbial population of the inoculants. The microbial population was monitored for two consecutive years, four times each during the growing season. Hourly air temperature and rain intensity data were retrieved from a weather station belonging to the Finnish Meteorological Institute, which is 3 km away from the experimental site. The first experiment aimed to find out whether the added inoculants could survive under rooftop conditions, and what the microbial development patterns were. The second experiment was conducted in a growth chamber using substrates with two different pH, which were collected from the balcony gardens of a residential building in Helsinki. *T. repens* and *V. tricolor* growing from seeds were cultivated in lab conditions for two months before quantifying *R. irregularis* colonization level. The second experiment aimed to test the viability of *R. irregularis* in the balcony garden substrate, and the effect of substrate pH on *R. irregularis* colonization level. The third experiment was conducted on the rooftop of a concrete factory hall. *F. vesca* and *T. serpyllum* were

inoculated with *R. irregularis*, with or without biochar amendment in the substrate, which aimed to investigate the effect of biochar amendment on *R. irregularis* colonization level (Fig 5).

The second publication (**II**) was conducted in lab conditions. Plant seeds (*A. dioica*, *C. rotundifolia*, *F. vesca*, *G. sanguineum*, *L. corniculatus*, *T. serpyllum*, *T. repens*, and *V. tricolor*) were kept four weeks in a growth chamber for germination. Afterward, the seedlings were singe- and co-inoculated with *R. irregularis* and *B. amyloliquefaciens* when they were transferred into a high-throughput phenotyping infrastructure called NaPPI (National Plant Phenotyping Infrastructure). NaPPI can control growing conditions and automatically monitor plant growth using specific cameras. The plants were kept at favorable growing conditions for another seven weeks before collecting root, shoot and substrate samples. The root and substrate samples were collected to detect *R. irregularis* colonization level and *B. amyloliquefaciens* bacterial density, respectively. The shoot samples were oven-dried and weighed to evaluate the effect of single- and co-inoculation on plant growth (Fig 5).

The third publication (**III**) was conducted on open vegetated roofs. The eight plant species tested in the second publication (**II**) were introduced on the vegetated roof with three planting methods: pre-grown vegetation mat, pre-grown plug plants, and seeds. Later, the microbial inoculants of *R. irregularis* and *B. amyloliquefaciens* were applied twice (with a one-month interval) onto the experimental fields to ensure successful inoculation. The vegetated roofs were minimally maintained by experienced gardener according to local weather conditions. Ten weeks after the first inoculation, root, shoot and growth substrate samples were collected from treated and control plots. *R. irregularis* colonization level and *B. amyloliquefaciens* bacterial density were measured, as well as plant growth (Fig 5).

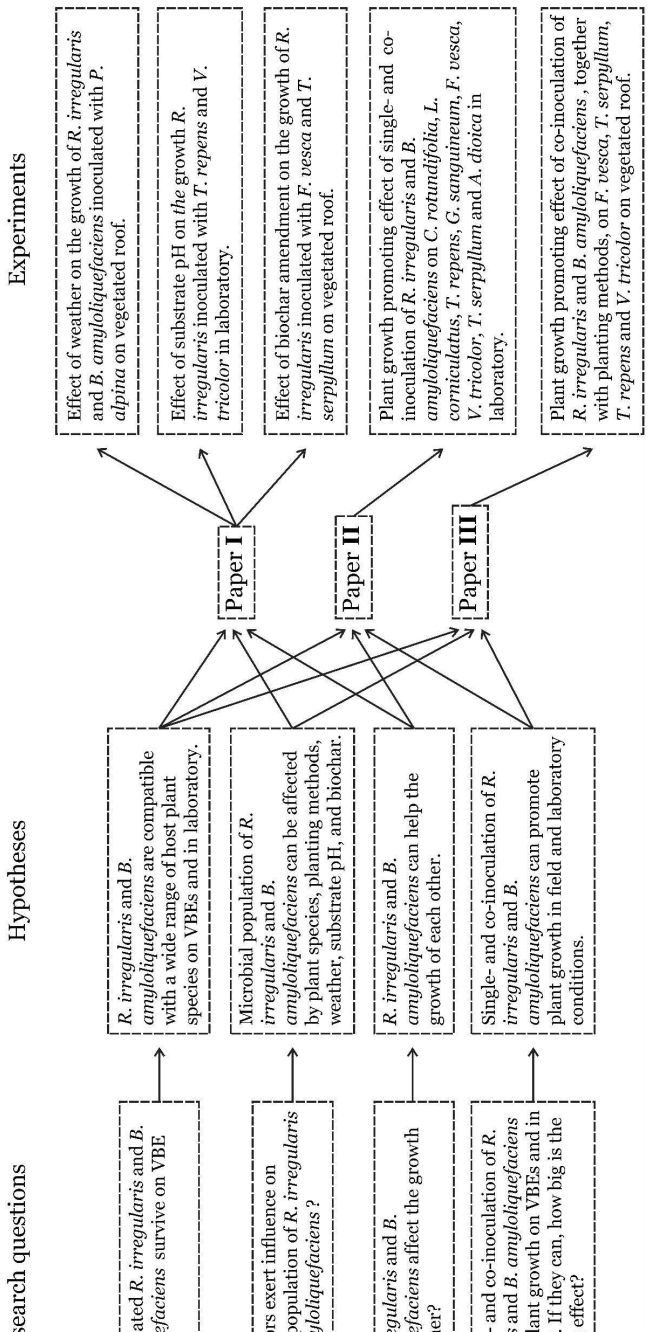

Fig 5. Flowchart of the research project from research questions and hypotheses to experiments.

4. Results and discussion

4.1 *R. irregularis* and *B. amyloliquefaciens* can colonize a wide range of plant species (I, II, III)

When *R. irregularis* and *B. amyloliquefaciens* were inoculated with nine different plant species, almost all the plants showed compatibility in both single- and co-inoculation. Only *C. rotundifolia* and *P. alpina* showed minimal or no trace of *R. irregularis* colonization. More importantly, most plants exhibited elevated levels of *R. irregularis* colonization when *B. amyloliquefaciens* was co-inoculated (details in 4.3).

It suggests that both *R. irregularis* and *B. amyloliquefaciens* are compatible with a wide range of plant species. Lack of host-plant specificity in AMF might be related to the continuous underground hyphal web that connects nearby plants and allows nutrients flow within the plant community. Additionally, without host specificity, AMF could colonize a wide range of plant species, which ensures a higher success (Sanders, 2003). *B. amyloliquefaciens* was also found to be compatible with a wide range of plant species (Santoyo and Orozco-Mosqueda, 2012; Chowdhury et al., 2015), but the underlying reasons remain mostly unstudied. However, it was found that *B. amyloliquefaciens* resides and propagates at a higher density on root hairs, which might be due to abundant root exudates (Fan et al., 2012).

In conclusion. the low host specificity of *R. irregularis* and *B. amyloliquefaciens* makes them useful and efficient substrate additives for a wide range of plant species to promote plant growth in general cultivation practices.

4.2 Effect of growth factors on the microbial growth (I, II, III)

4.2.1 Plant species influenced *R. irregularis* colonization level in the field and lab (I, II, III)

In this project, nine different plant species were tested, two of which, i.e., *P. alpina* and *C. rotundifolia*, did not form mycorrhiza with *R. irregularis* (Table 2). *C. rotundifolia* could not be colonized by *R. irregularis* in the lab. Similarly, *P. alpina* was not colonized by *R. irregularis* on the vegetated roof. It suggests that *P. alpina* and *C. rotundifolia* may not be suitable hosts for *R. irregularis*. Firstly, *P. alpina* and *C. rotundifolia* are highly stress-tolerant plant species, which are less demanding for growth-promoting (Steiner et al., 2012; Stevens and Wilson, 2012). By not forming AMF symbiosis, they can save up to 20% of photosynthetic products, which is needed to host AMF (Wright et al., 1998). Secondly, unsuccessful *R. irregularis* colonization in *P. alpina* and *C. rotundifolia* might be attributed to the failed recognition process. Maybe their root exudates are not attractive to *R. irregularis*, or *R. irregularis* failed to bypass the innate immune system of *P. alpina* and *C. rotundifolia*. However, *P. alpina* and *C. rotundifolia* can still be colonized by some other mycorrhizal species. Kytöviita et al. (2003) found that *C. rotundifolia* was successfully colonized by three *Glomus* species (AMF), but only one exhibited a growth-promoting effect. *P. alpina* is also reported to be a host plant for mycorrhizal fungi in natural settings, but the species were not specified (Cripps and Eddington, 2005).

The other seven plant species were found compatible with *R. irregularis* at various levels in lab and field conditions (Table 2). Single-inoculation of *R. irregularis* exhibited constantly low colonization than co-inoculation with both *R. irregularis* and *B. amyloliquefaciens*. It implies that other factors are influencing *R. irregularis* colonization apart from plant species, which is elaborated in the following sections.

Table 2. The abundance of AMF structures in the eight tested plant species.

	Hypha abundance (%)							
	II		**III** (mat)		**III** (plug)		**III** (seed)	
Tested plants	Single	Dual	E roof	W roof	E roof	W roof	E roof	W roof
A. dioica	46.3	82.0	/	/	/	/	/	/
C. rotundifolia	0	3.0	/	/	/	/	/	/
G. sanguineum	3.0	48.0	/	/	/	/	/	/
F. vesca	6.7	95.0	37.0	20.0	57.0	30.2	/	/
L. corniculatus	0	40.0	/	/	/	/	/	/
T. repens	0	65.7	9.3	8.5	13.5	12.0	32.0	29.0
T. serpyllum	36.7	87.7	13.0	11.5	42.5	22.0	61.8	47.3
V. tricolor	17.7	46.0	/	/	/	/	52.5	56.0
	Arbuscule abundance (%)							
	II		**III** (mat)		**III** (plug)		**III** (seed)	
Tested plants	Single	Dual	E roof	W roof	E roof	W roof	E roof	W roof
A. dioica	13.3	63.3	/	/	/	/	/	/
C. rotundifolia	0	1.0	/	/	/	/	/	/
G. sanguineum	0	38.3	/	/	/	/	/	/
F. vesca	2.7	82.7	15.7	5.2	51.0	23.2	/	/
L. corniculatus	0	30.7	/	/	/	/	/	/
T. repens	0	58.0	3.5	3.7	32.7	7.2	10.5	19.7
T. serpyllum	18.7	47.0	8.0	3.8	7.7	14.5	43.5	32.5
V. tricolor	11.7	29.7	/	/	/	/	37.2	41.0
	Vesicle abundance (%)							
	II		**III** (mat)		**III** (plug)		**III** (seed)	
Tested plants	Single	Dual	E roof	W roof	E roof	W roof	E roof	W roof
A. dioica	7.3	14.0	/	/	/	/	/	/
C. rotundifolia	0	0	/	/	/	/	/	/
G. sanguineum	0	8.7	/	/	/	/	/	/
F. vesca	0	14.0	8.7	2.0	4.5	3.2	/	/
L. corniculatus	0	11.0	/	/	/	/	/	/
T. repens	0	21.3	0	0	0	0	0	0
T. serpyllum	2.0	8.0	0	0	0	0	0	0
V. tricolor	0	1.3	/	/	/	/	2.3	1.2

Single: single-inoculation with *R. irregularis* in paper **II**;
Dual: co-inoculation with *R. irregularis* and *B. amyloliquefaciens* paper **II**;
E roof: eastern roof in paper **III**;
W roof: western roofs in paper **III**;
/: not available.

Sanders (2003) concluded that AMF symbiosis has been widely acknowledged as non-species specific interaction between the fungi and host plants, meaning a given AMF species can colonize a certain range of plant species and vice versa. It is a beneficial strategy that would ensure more successful and higher mycorrhizal colonization. However, lacking specificity does not necessarily suggest that there is no selection pressure on both parties. AMF have been found to colonize certain plants more efficiently than the others, resulting in different colonization level. The difference in AMF compatibility may be associated with root exudate composition and nutrient absorption ability of different plant species (Legay et al., 2016).

4.2.2 Plant species influenced *B. amyloliquefaciens* bacterial density in the field (III), but not in the lab (II)

Plant species were found to affect *B. amyloliquefaciens* bacterial density only on vegetated roofs, but not in the lab. In the vegetated roof experiment (**III**) in which *B. amyloliquefaciens* was inoculated in the non-sterile substrate, ANOVA revealed that *B. amyloliquefaciens* resided in the rhizosphere of *T. repens* at a significantly higher density than *F. vesca*, *T. serpyllum*, and *V. tricolor*. While in lab experiment (**II**) in which *B. amyloliquefaciens* was inoculated in the sterile substrate, no such difference was detected among all tested plant species (including *F. vesca*, *T. repens*, *T. serpyllum*, and *V. tricolor*).

The contradictory results might be attributed to the presence of the microbial community in substrates. Pantastico-Caldas (1992) found that a virulent phage and *B. subtilis* exhibited a co-oscillation in their microbial population. Young (1995) also found out that *Bacillus cereus* was affected by the presence of other microbes in the substrate. Such an effect might be caused by either microbial competition, antagonism or synergy. Growing in the non-sterile substrate, plants could affect the bacterial density of *B. amyloliquefaciens* by shaping the microbial community and their function through species-specific root exudates. In autoclaved substrate, plants could not exert such influence through exudates (Berg and Smalla, 2009).

Moreover, different growing conditions between lab and rooftop might also lead to such outcomes. Plants were maintained in a more preferable growing condition in the lab than on the vegetated roofs. It has been reported that stressed conditions would stimulate plants to release chemoattractant-containing exudates to lure PGPMs, such as malic acid (Keeley, 1978; Henry et al., 2007). Malic acid has been found to stimulate the growth of *B. subtilis* (a PGPR species closely related to *B. amyloliquefaciens*) and its biofilm formation (Rudrappa et al., 2008; Chen et al., 2012). Therefore, plants would proactively lure and support PGPMs at a higher density in response to stressors.

4.2.3, Planting methods affected *R. irregularis* colonization level, but not *B. amyloliquefaciens* bacterial density in the field (III)

According to the result from **III**, the planting method had a significant effect on *R. irregularis* colonization level, which exhibited the following patterns: seed > plug > mat. Host plant age may be the underlying reason of why seed-grown plants are more effectively colonized by *R. irregulars* than pre-grown plug and mat plants. It has been found that mycorrhization is more welcomed by younger plants. For one thing, young plants produce fine roots with a thin cell wall, which makes it easier for AMF hyphae to penetrate and spread (Wilcox, 1983; Sohn et al., 2003). For another, mature plants might alter root exudates that are less attractive to AMF to establish symbiosis than seedlings (Buee et al., 2000; Buée et al., 2009; Micallef et al., 2009). In this experiment, pre-nursery plants in the mat and plug planting methods were fully grown before installing on the vegetated roofs. Therefore, these mature plants were less colonized by *R. irregularis* than young plants growing from seeds.

Moreover, the reason why plug plants were more colonized than mat-grown plants might be because of plant/root density. In the high root density situation, the amount of AMF spores in the substrate can be a limiting factor for a higher colonization level (Koide and Dickie, 2002). Plug plants (16 per m^2) were more sparsely distributed than mat-grown plants (50-150 plants per m^2). Competition

for *R. irregularis* colonization was less intense for plug plants than for mat-grown plants, resulting in a higher colonization level than the later.

Additionally, since the inoculation was conducted by spreading solutions containing the inoculants, plant foliage might have blocked the inoculant solution from reaching the substrate and plant roots. When applying the inoculant solution, the vegetation coverage were mat > plug > seed. So, the chance and amount of inoculant solution reaching to the ground would be seed > plug > mat, which is consistent with the order of AMF colonization level.

On the contrary, planting methods did not show any effect on the bacterial density of *B. amyloliquefaciens*. As described in the former section, cells of *B. amyloliquefaciens* reside in the rhizosphere, which means no root surface penetration is required. In that sense, root structure, determined by plant age, was a less limiting factor for *B. amyloliquefaciens* than for *R. irregularis*. Furthermore, host plants must compete with others to attract AMF spores to establish an interaction. During the competition, AMF spores may have become a limiting resource. However, *B. amyloliquefaciens* can simply reproduce in the rhizosphere to meet the needs, even if the initial amount of *B. amyloliquefaciens* in the substrates was reduced by foliage blockage in the mat and plug planting methods. For example, in a greenhouse experiment, *Triticum aestivum* was inoculated with *Azospirillum brasilense* (a PGPR) either once on day 8 (single inoculation) or 4 times from day 8 with an 8-day interval (successive inoculation). By day 40, bacteria density of *A. brasilense* did not differ between single and successive inoculation in the rhizosphere of *T. aestivum* (Bashan, 1986). It suggests that an initial small amount of PGPR propagules in the rhizosphere does not limit the growth of the bacteria. Theoretically, there will be no limited *B. amyloliquefaciens* cells, as long as nearby plants can produce adequate exudates for *B. amyloliquefaciens* to grow and propagate.

4.2.4 Weather conditions affected *B. amyloliquefaciens* bacterial density in the field (I)

The experiment was aimed to test the effect of weather conditions on both *R. irregularis* and *B. amyloliquefaciens* inoculated to *P. alpina*. However, *R. irregularis* was not detected in *P. alpina*. So, the effect of weather will only focus on *B. amyloliquefaciens* bacterial density. A portable weather station was used to measure substrate temperature and substrate moisture on site, but the data was incomplete due to equipment missing. So, air temperature and precipitation data were retrieved from Finnish Meteorological Institution to represent the growing conditions on the rooftop.

In 2012, the temperature was mild, and precipitation was adequate during the experimental period (Fig 5a in **I**). The *B. amyloliquefaciens* bacterial density during 2012 experienced an exponential growth (Fig 4a in **I**). In contrast, the weather became hotter and drier in 2013, especially in the later half period of the experiment (Fig 5b in **I**). Consequently, *B. amyloliquefaciens* bacterial density decreased dramatically from the beginning to the end of the measurement period in 2013 (Fig 4b in **I**).

In a study where *B. amyloliquefaciens* DL-3 was cultivated under three different substrate temperature conditions: 32, 37, and 42°C, it proliferated at a higher population under 32°C than 37 and 42°C (Jo et al., 2008). Other studies also suggested an optimal temperature range for various *Bacillus* species is between 27 and 37°C, and their vitality was impaired when substrate temperature surpassing 45°C (Yoon et al., 2001; Aslim et al., 2002; Ahmed et al., 2007). According to the incomplete data from the portable weather station, in 2013, even if air temperature seldom exceeded 30°C, soil temperature could easily reach over 40°C. In extreme cases, soil temperature was 52°C when the air temperature was about 31°C.

Besides temperature, water availability can also affect *Bacillus* bacterial density. Vardharajula et al. (2011) found that bacterial density of *B. amyloliquefaciens* reduced to 1/10 cultivated at 46.6% water holding capacity (WHC) compared to 75% WHC. In the latter half of the experiment in 2013, lack of rain for 1.5 months

created drought stress for *B. amyloliquefaciens*, which could greatly reduce its bacterial density.

Cocking (2003) pointed out that epiphytic bacteria (living on root surfaces) are vulnerable to competition from other substrate microbes. It is assumed that as an epiphytic bacterium, *B. amyloliquefaciens* is also vulnerable to other stresses in the substrate, such as drought and heat. However, studies are still rare in finding out the responses and sensitivity of PGPMs towards drought and heat events.

In conclusion, a combination of substrate temperature and substrate moisture might be the key factor that *B. amyloliquefaciens* bacterial density exhibited a steady increase in 2012 when the climate was mild and steady decrease in 2013 when the weather was hot and dry.

4.2.5 Effect of substrate pH and biochar amendment on *R. irregularis* colonization level (I)

The effect of substrate pH and biochar amendment were tested on colonization level of *R. irregularis*, but not on the bacterial density of *B. amyloliquefaciens*, due to field space limitation and narrow focus of experiment designed by other group members. Thus, *B. amyloliquefaciens* was not monitored in substrate pH and biochar amendment experiments. The effect of substrate pH on *R. irregularis* colonization was tested on *T. repens* and *V. tricolor*, and the effect of biochar amendment was tested on *F. vesca* and *T. serpyllum*.

In the substrate pH experiment, *T. repens* showed a higher colonization level in the mildly acidic substrate (pH 6-6.5) than in the acidic substrate (pH 5-5.5). On the contrary, *V. tricolor* in acidic substrate (pH 5-5.5) exhibited a higher colonization level, compared with *V. tricolor* in the mildly acidic substrate (pH 6-6.5). According to ANOVA, the colonization level of *R. irregularis* was significantly affected by plant species, substrate pH, and their interactions (**I**). Another study found that AMF is sensitive to substrate pH, and different AMF species have different suitable pH ranges. The authors used *Vigna unguiculata* as the host plant and showed that AMF species *Glomus etunicatum* significantly increased its

colonization level when substrate pH rose from 4.7 to 5.2. While, the substrate pH change did not show any significant effect on the *Gigaspora margarita* colonization level (Rohyadi et al., 2004). These findings suggest that the mycorrhization level can be affected by substrate pH, plant species, mycorrhizal species, and their interactions.

Plants would produce and accumulate reactive oxygen species (ROS) under stressed conditions, including unsuitable substrate pH (Shi et al., 2006; Zhang et al., 2017), and ROS would inhibit colonization efficiency of AMF (Lenoir et al., 2016). Thus, the AMF colonization level is determined by host plant tolerance to substrate pH. For instance, it was found that *T. repens* produced higher biomass in substrate pH at 6.5 than 6 and below (Deska et al., 2011). And when grown in the acid substrate (pH 4-5), *T. repens* would increase rhizospheric substrate pH to a higher level as a strategy to evade acidic stress (Snaydon, 1962). It is surmised that *T. repens* in the present study was stunted at 5-5.5 substrate pH, and accumulated ROS which inhibited *R. irregularis* colonization. The inhibition was not present when *T. repens* grew in a less acidic substrate, and consequently, AMF colonization increased. On the other hand, it is reported that *Viola* × *wittrockiana*, derived from *V. tricolor* in central Europe, grew best at substrate pH 5.4 to 5.8. Substrate pH above 5.8 can result in boron and iron deficiency and may also lead to an increased incidence of black root rot (Bailey, 1998). In that sense, acidic substrate outperforms neutral/alkaline substrate in *V. tricolor* cultivation and support a higher level of *R. irregularis* colonization in the roots.

As for biochar effect on mycorrhization, a negative impact was found between the biochar amendment and *R. irregularis* colonization. In the present study, *F. vesca* and *T. serpyllum* plants without the biochar amendment demonstrated a higher mean colonization level than biochar-amended plants. Even though the difference was not statistically significant, the p values of biochar effect on hypha and arbuscule abundance (0.090 and 0.081 respectively) were close to the threshold of 0.05 according to ANOVA (Table 3 in **I**).

According to literature, the effect of biochar on AMF growth can be either positive, negative, or neutral (Koide, 2017). There are two mechanisms through which biochar exerts influence on mycorrhization. Firstly, biochar can modify substrate

property, which indirectly affects the AMF colonization level. For instance, biochar has been found to increase P availability, whose inhibitory effect on mycorrhization has been extensively studied and revealed (Nouri et al., 2014; Koide, 2017). Biochar provides porous surfaces for substrate microbes and increases water holding capacity to alleviate drought stress (Warnock et al., 2007). Biochar can also increase pH in the acid substrate by alkaline ash residual on the surface (DeLuca et al., 2015). The shift in substrate pH can influence mycorrhization as explicitly explained in earlier paragraphs. Secondly, biochar can influence other microbes that might affect mycorrhization, such as mycorrhiza helper bacteria (MHB) and phosphate solubilizing bacteria (Pietikäinen et al., 2000; Warnock et al., 2007; Lehmann et al., 2011). These mechanisms have either positive or negative effects on mycorrhization and collectively contribute to the outcome. Despite the contradictory findings from existing studies, biochar is generally regarded as a beneficial amendment in the substrate and has the potential to apply in VBEs substrates (Koide, 2017).

4.3 *B. amyloliquefaciens* acted as mycorrhiza helper bacterium of *R. irregularis* (II)

Co-inoculation of *R. irregularis* and *B. amyloliquefaciens* in sterile substrates in lab conditions significantly increased *R. irregularis* colonization level in seven out of the eight tested plant species. The abundance of hypha increased from 0-50% in single-inoculation to 40-100% in co-inoculation; arbuscule from 0-20% to 30-80%; and vesicle from 0-10% to 0-25% (Table 2).

According to the effect of co-inoculation on *R. irregularis* colonization level, the eight plant species can be divided into three groups: 1) non-host plants regardless the presence of *B. amyloliquefaciens* (*C. rotundifolia*); 2) facultative host plants when co-inoculated with *B. amyloliquefaciens* (*L. corniculatus* and *T. repens*); 3) host plants that were more colonized in co-inoculation than *R. irregularis* single-inoculation (*A. dioica*, *F. vesca*, *G. sanguineum*, *T. serpyllum*, and *V. tricolor*). The results qualified *B. amyloliquefaciens* as MHB for *R. irregularis*. The idea of MHB was firstly proposed by Duponnois and Garbaye (1991) when they observed a

significant increase of ectomycorrhizal formation when co-inoculated with *Pseudomonas fluorescens* BBc6. Since then, many bacteria have been identified as MHB, and more MHB will likely be revealed in the future (Artursson et al., 2006).

The effect of MHB has been reported to be achieved via direct and indirect mechanisms (Deveau and Labbé, 2016). Direct mechanism refers to the stimulation of the AMF growth directly by releasing soluble and volatile compounds. For instance, gaseous volatiles (2-methylisoburneol and acetoin) and nutrients (vitamins, amino acids, and growth substances) released by MHB could regulate and stimulate the growth of AMF (Kai et al., 2009). The indirect mechanism refers to the modification of host plants by MHB in favor of AMF colonization. For instance, some MHB could increase lateral root number which can be easily colonized by AMF, increasing the total colonization level (Poole et al., 2001; Bending et al., 2002). Many MHB, such as *B. amyloliquefaciens*, are also identified as PGPMs which not only facilitate mycorrhizal colonization but also improve the growth of their common host plants (Deveau and Labbé, 2016).

It exhibited from the present study that *B. amyloliquefaciens* is obligatory in *R. irregularis* colonization for legumes, i.e., *L. corniculatus* and *T. repens*, suggesting that for certain plant species, a successful AMF colonization is strongly dependent on MHB. It has been revealed that legumes and their nodulation are associated with unique AMF community, which is different from nonlegumes (Scheublin et al., 2004). The specific legume-AMF association might be attributed to specific nutritional requirements of legumes or host-specific interaction. A prior study also found that nodule-inducing rhizobia *Bradyrbizobium japonicum* enhanced colonization of *Glomus mosseae* (an AMF species) in *Glycine max*. The author suggested that *B. japonicum* could produce an acetylated nodulation factor, leading to the accumulation of soybean flavonoids, and the increased flavonoids further meditate mycorrhizal colonization in the roots (Xie et al., 1995). Such mycorrhiza helping effect was also determined by the timing of endophyte inoculation: early *G. mosseae* inoculation and late *B. japonicum* inoculation promoted fugal development of *G. mosseae*, and late *G. mosseae* inoculation and early *B. japonicum* inoculation had the opposite effect (Bethlenfalvay et al., 1985).

Besides legumes, nonlegumes also exhibited specific AMF promotion in the tripartite association (rhizobacteria, AMF, and host plants). In an examination of plant growth parameters of *Cucumis sativus* which is co-inoculated with different combinations of rhizobacteria and AMF, certain *Paenibacillus* strains could positively or negatively affect *G. intraradices* and *G. mosseae* colonization, while the other *Paenibacillus* strains could not (Li et al., 2008). Garbaye and Duponnois (1993) found that the helping effect of MHB isolates on *Laccaria laccata* S238 (an ectomycorrhizal fungus) was not affected by conifer plant species, but by the combination of mycorrhizal fungal species and MHB isolates.

In conclusion, *B. amyloliquefaciens* inoculation significantly increased colonization level of *R. irregularis* for a group of different host plant species, and such a helping effect may be dependent on microbial combinations, but not host plant species.

4.4 Can *R. irregularis* in return promote the growth of *B. amyloliquefaciens* (I, II)?

In this project, the bacterial density of *B. amyloliquefaciens* was not affected by the co-inoculation of *R. irregularis* among the eight plant species when cultivated in sterile and lab conditions (**II**). However, when *R. irregularis* and *B. amyloliquefaciens* were separately inoculated on the vegetated roof, *B. amyloliquefaciens* bacterial density in *R. irregularis* treated plots was more than 600 times higher than in non-inoculated control plots, even though no *B. amyloliquefaciens* was applied in *R. irregularis* treated plots (**I**). This result indicated that *R. irregularis* might promote the growth of *B. amyloliquefaciens,* which was a local strain in the unsterilized vegetated roof substrates.

It has been well established that plants can attract or repel substrate microbes through specific root exudates (Huang et al., 2014). Additionally, AMF colonization could also exert selection pressure on bacteria in mycorrhizosphere. Some bacterial species (especially gram-positive bacteria) tend to associate with AMF, resulting in a higher bacterial density in mycorrhizosphere (Miransari, 2011).

For instance, bacterial species *Paenibacillus brasilensis* (an N-fixing bacterium) and *P. fluorescens* (a pathogen resistant bacterium) were more attracted to AMF species *Glomus* sp. than *B. cereus*, *Arthrobacter chlorophenolicus*, and *Paenibacillus peoriae*, which are also PGPR. It suggested that such bacteria-AMF association is dependent on bacterial and fungal species (Toljander et al., 2006).

Furthermore, stresses would induce exudate change from mycorrhizal plants, which further influence the bacterial community. In a study inoculating *G. mosseae* to treat wilt disease caused by *Fusarium oxysporum*, researchers found that AMF colonization altered root exudates by increasing allelopathic substances that antagonize pathogens. Malic acid was one of the substances exhibited a significant increase in *Citrullus lanatus* plants colonized by *G. mosseae* than control ones (Ren et al., 2015). Malic acid was found to stimulate the growth and biofilm formation of *Bacillus* species (Rudrappa et al., 2008; Chen et al., 2012). It was also reported that malic acid could induce the swarming motility and population density of a PGPR called *Paenibacillus polymyxa* (Ling et al., 2011).

In this project, drought and heat stress on the vegetated roof might trigger mycorrhizal plants to produce chemical signals and attractants in root exudates to lure and support *B. amyloliquefaciens* to harvest its growth-promoting effect. As a result, the density of *B. amyloliquefaciens* was increased in the mycorrhizosphere. While in the lab, no stresses would induce such exudate alteration. Therefore, *B. amyloliquefaciens* bacterial density was not affected by mycorrhization.

4.5 Plant growth-promoting effect of *R. irregularis* and *B. amyloliquefaciens* (II, III)

4.5.1 Single-inoculation of *B. amyloliquefaciens* promoted plant growth, but single-inoculation of *R. irregularis* did not in the lab (II)

In the second study from the greenhouse, *B. amyloliquefaciens* was detected in sterile substrates of all the tested plant species inoculated with *B.*

amyloliquefaciens, but not in control. And single-inoculation of *B. amyloliquefaciens* in sterile substrates produced a plant growth-promoting effect in all the tested plant species.

In contrast, *R. irregularis* did not exhibit any promoting effect on plant growth when it was single-inoculated in sterile substrates, even though *R. irregularis* colonization was confirmed in some of the plant species (Fig 3 in **II**). Failure in promoting plant growth in *R. irregularis* single-inoculation might be attributed to 1) insufficient AMF colonization, 2) other benefits other than plant growth-promoting, and 3) loss of the promoting effect of AMF species in the sterile substrate.

R. irregularis colonization was barely confirmed in *C. rotundifolia*, with or without MHB. It suggested that *C. rotundifolia* might not need *R. irregularis* symbiosis for growth-promoting as it is a stress-tolerant plant species (Steiner et al., 2012; Stevens et al., 2012). Moreover, mycorrhizal plants *G. sanguineum*, *L. corniculatus*, and *T. repens* also showed nearly no colonization, which might be caused by lacking MHB in sterile substrates (section 4.3). Due to insufficient AMF colonization, plant growth was not enhanced for the four plant species in single-inoculation.

On the other hand, *A. dioica*, *F. vesca*, *T. serpyllum*, and *V. tricolor* showed various levels of colonization in *R. irregularis* single-inoculation: 5-50% of hypha abundance and 3-20 % of arbuscule abundance. Yet, there was no trace of plant growth-promotion neither. *R. irregularis* might provide benefits other than biomass increase, which was not checked in the present study. Therefore, plants could still welcome mycorrhization even without visible growth promotion (Sanders, 2003). For instance, *Lolium perenne* and *Anthyllis vulneraria* plants were inoculated with AMF inoculant mixture in sterile substrates to study the impact of AMF colonization on substrate erosion from wind. Both plant species were successfully colonized. *L. perenne* reduced ¼ of aboveground biomass in the mycorrhizal treated group, while *A. vulneraria* did not show any change. However, a significant reduction in substrate loss caused by the wind was detected for both plants that were inoculated with AMF (Burri et al., 2013).

In another study conducted by Kloepper and Schroth (1981), the inoculation of PGPR in the sterile substrate did not promote the growth of *Raphanus raphanistrum*. When PGPR was inoculated in the non-sterile substrate, the plant growth was significantly increased by 50-150%. Associating the facts that both substrates in the present study and Burri's study were sterile, the loss of AMF plant growth-promotion might be the result of lacking interaction between the AMF and local microbial community (Kloepper and Schroth, 1981).

In conclusion, successful AMF colonization does not guarantee plant growth-promotion, and interaction between AMF inoculants with local microbial community plays an important role in plant growth-promoting effect by AMF.

4.5.2 Co-inoculation of *R. irregularis* and *B. amyloliquefaciens* produced higher plant growth than their single-inoculation in the lab (II)

In the second study from the greenhouse, co-inoculation of *R. irregularis* and *B. amyloliquefaciens* produced higher plant growth than single-inoculations for *A. dioica*, *F. vesca*, *G. sanguineum*, *L. corniculatus*, *T. repens*, and *V. tricolor*, but not for *C. rotundifolia* and *T. serpyllum* (**II**). It suggests that the co-inoculation of *R. irregularis* and *B. amyloliquefaciens* could enhance the growth of certain plant species, and the promoting efficiency of the co-inoculation was dependent on plant species.

According to Burri et al. (2013) and Kloepper and Schroth (1981), such a promoting effect of the co-inoculation might be further enhanced using non-sterile substrates. In another study utilizing *Bacillus velezensis* Bs006 as an inoculant to control Fusarium wilt on *Physalis peruviana* plants in both sterile and non-sterile substrate, the severity of the wilt symptom exhibited such pattern: sterile substrate+*B. velezensis* Bs006 > non-sterile substrate > non-sterile substrate+*B. velezensis* Bs006. The result implied that local microbial community without *B. velezensis* Bs006 possess a certain level of pathogen resistance, and pathogen resistance of *B. velezensis* Bs006 was significantly elevated by interacting with local substrate microflora (Moreno-Velandia et al., 2019). All the results indicated

that PGPR inoculants could enhance plant growth-promotion by interacting with the substrate microbial community.

The results from the present and previous studies put forward the necessity of investigating the beneficial interaction between PGPM inoculants and the local microbial community. Plant cultivation and production in various ecological systems would benefit from revealing the underlying mechanisms of the interactions that lead to higher synergistic effects.

4.5.3 Co-inoculation of *R. irregularis* and *B. amyloliquefaciens* promoted plant growth in the field, but not as much as in the lab (II, III)

According to the present project, most co-inoculated plants significantly increased their shoot biomass than non-inoculated control plants on vegetated roof conditions, and plant growth was less enhanced by co-inoculation in the field than in the lab (Table 3). *F. vesca* increased 648 and 578% in the lab, but only increased less than 100% on vegetated roofs. *T. repens* has recorded the biggest increase in the lab of 2447%, but on vegetated roofs, the increase did not exceed 100% either. *T. serpyllum* exhibited the smallest increase of 388% in the lab, but still much higher than that in the vegetated roofs, which was between 75 and 95%. The increases in *V. tricolor* were the highest in vegetated roof conditions (292 and 223%), but only about half the increase recorded from the lab (Table 3).

Table 3. Increase in dry aboveground biomass of co-inoculated plants compared with non-inoculated control plants in both lab conditions (**II**) and field conditions (**III**).

	Biomass increase (%)							
	II		**III** (mat)		**III** (plug)		**III** (seed)	
Tested plants	Exp. 1	Exp. 2	E roof	W roof	E roof	W roof	E roof	W roof
F. vesca	648	578	72.1	66.9	98.5	53.5	/	/
T. repens	717	2447	18.1	64.1	32.4	40.6	36.8	27.4*
T. serpyllum	1883	388	84.0	88.7	75.6	88.8	75.6	94.7
V. tricolor	579	712	/	/	/	/	292	223

Exp. 1: the first greenhouse experiment in paper II;
Exp. 2: the second greenhouse experiment in paper II;
*: the increase that did not exhibit a significant difference.

Compared with lab conditions, outdoor vegetated roof conditions are usually less stable and less suitable. As a result, plants tend to grow smaller on vegetated roofs than in the lab. Therefore, even though no studies compared plant growth-promoting efficiency of AMF under roof and lab conditions, roof conditions may be analogized to biotic or abiotic stresses. In general, AMF-inoculated plants without stress (in the lab with AMF) outgrow AMF-inoculated plant in stress (on the rooftop with AMF), and AMF-inoculated plants in stress outgrow non-mycorrhizal plants under the same stress (on the rooftop without AMF). For instance, it was found that leaf number and leaf area of *V. unguiculata* plants followed the pattern: AMF+watering > AMF+water stress > water stress (Oyewole et al., 2017).

It is assumed that in stressed conditions, AMF distributed its energy to induce plant resistance against such stress, so its growth-promoting function was curtailed. As a result, plant growth-promotion by AMF in stressed conditions was less prominent than stress-free conditions when AMF distributes its entire energy in plant growth-promotion.

5. Conclusions and prospects

The project consists of three stepwise studies. Firstly, *R. irregularis* and *B. amyloliquefaciens* were inoculated in VBEs to find out their ability to survive in VBE growing conditions, and factors that could affect their survival (**I**). Secondly, *R. irregularis* and *B. amyloliquefaciens* were inoculated with different plant species in the lab to find out suitable hosts, and the compatibility of the two microbes (**II**). Thirdly, *R. irregularis* and *B. amyloliquefaciens* were inoculated on the vegetated roof to test if the effects observed in the lab could repeat in the field (**III**).

The most important finding is that *B. amyloliquefaciens* acted as MHB to promote *R. irregularis* colonization, and the promoting effect of co-inoculation on plant growth occurred for most of the tested plant species in both lab and field. The present project also investigated the factors that affect the microbial density of the inoculated PGPMs, such as weather conditions, biochar amendment, substrate pH, and planting method, which have been seldom studied on VBEs (Fig 6).

According to the findings, when constructing VBEs, it is suggested:
1) Using substrates and plant species that support PGPM inoculants, such as thicker substrate layers with proper pH and low nutrients (especially P and N);
2) Co-inoculating *R. irregularis* and *B. amyloliquefaciens* to achieve synergistic effects on plant growth;
3) Using vegetation mat and plug plants to achieve instant greening, as well as using seed-grown plants that favor AMF colonization;
4) Inoculating *R. irregularis* and *B. amyloliquefaciens* with mat and plug plants before installing on VBEs, so that the symbiosis will be established beforehand, and the efforts to apply the inoculants on VBEs will be spared;
5) Irrigating the plants moderately during prolonged heat and dry periods in the first few years, helping the plants and microbial community to survive, yet allowing them to adapt to the harsh rooftop conditions;
6) Avoiding microbes that are invasive species, which might pose threat to the local microbial community and consequently influence ecosystems and plant community (Van der Putten et al., 2007).

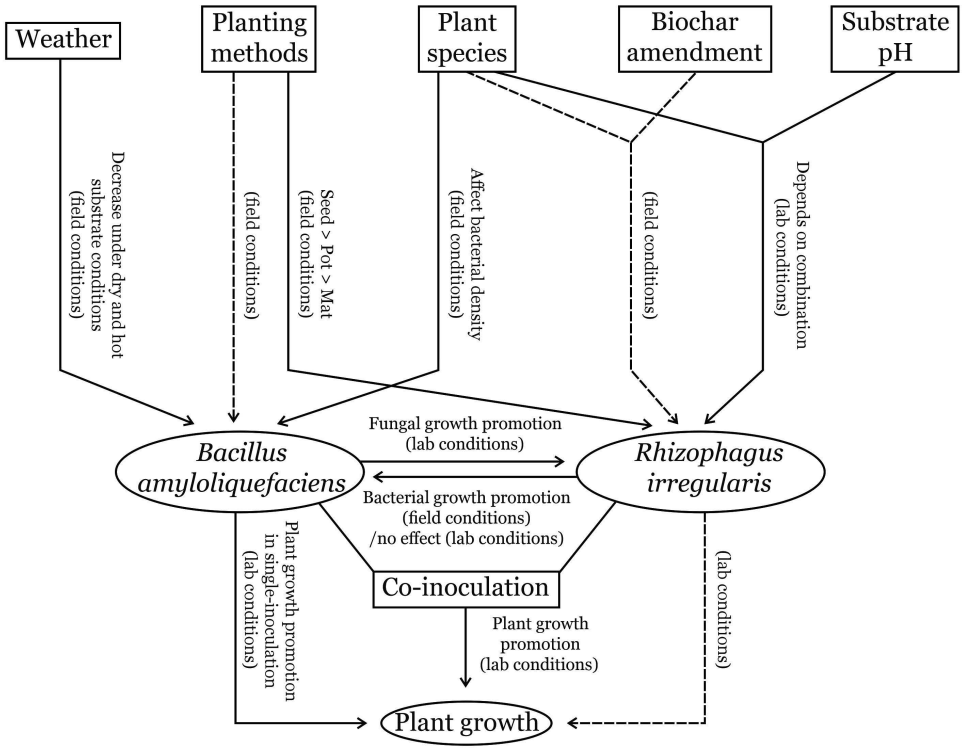

Fig 6. The effect of plant species, planting methods, weather conditions, substrate pH, and biochar amendment on the microbial population of *R. irregularis* and *B. amyloliquefaciens*, and on plant growth-promotion. A solid arrow indicates a significant effect. A dash arrow indicates no significant effect detected.

Still, some questions that were not answered in the present project. For instance, it was not sure whether *R. irregularis* could return the favor to promote the growth of *B. amyloliquefaciens*, and what are the factors affecting such promoting effect if there is any. So, the first prospect is to conduct a lab experiment using GFP tagging and confocal microscopy techniques to reveal the affinity of *B. amyloliquefaciens* to *R. irregularis* (Artursson and Jansson, 2006). GFP tagging and confocal microscopy techniques visualize clear attachment of bacteria on mycorrhizal hyphae and give a better clue of bacterial-mycorrhizal interaction. It would provide evidence and basis for further greenhouse and field experiments.

It was hypothesized that plant growth-promoting effect of *R. irregularis* and *B. amyloliquefaciens* were dependent on interaction with the local microbial

community in substrates, but there was no hard evidence from this project. Therefore, the second prospect is to explore the effect of the local microbial community on single- and co-inoculation of *R. irregularis* and *B. amyloliquefaciens*. On top of that, the plant community can be introduced as an additional factor.

Furthermore, the effect of PGPM inoculation in the substrates of living walls could be investigated. Besides their plant growth-promoting effect on living walls, other unique benefits can be focused on. For instance, it would be interesting to test whether PGPM could reduce substrate loss on outdoor living walls, which may be caused by wind erosion (Stein and Gestwa, 2015). A wind tunnel experiment revealed that mycorrhizal fungi could decrease wind erosion in lab conditions by stabilizing substrate with extraradical hyphae and fungal exudates (Burri et al., 2013). Additionally, another lab experiment exhibited that living walls can effectively remove volatile organic compounds (VOCs) in the air when forcing the air through the substrates and foliage (Torpy et al., 2018). It was suggested that bacteria in the living wall substrates have an important role in absorbing and degrading VOCs (Russell et al., 2014). It would be valuable to test if *R. irregularis* and *B. amyloliquefaciens* could provide air purification function when inoculated in such living walls.

6. References

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